

Retrograde DiI labeling

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An abbreviated version of this protocol was published in eLIFE in Oct 2017

Sparse genetic tracing reveals regionally specific functional organization of mammalian nociceptors

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Detailed protocol

Retrograde Dil labeling of L3-L5 DRGs

Solutions:

0.2 mg/mL Dil (Molecular Probes, Cat# D282) in DMSO/PBS

To prepare: Dissolve Dil crystals in DMSO to a concentration of 1mg/mL. Then, dilute 1:5 in sterile PBS, yielding a final concentration of 0.2 mg/mL

Subcutaneous Dil injection:

1. Prepare injection syringe:
 - a. Pipette 1 μ L of 0.2 mg/mL Dil solution onto a small piece of parafilm.
 - b. Remove cap from a sterile 29G, 0.5 inch insulin syringe, push plunger to expel any air in the syringe.
 - c. Slowly and carefully aspirate droplet of Dil solution into needle of insulin syringe. (It is important to gently pull up on the syringe just until the droplet sits in the needle, but does not become fully sucked into the syringe. If the droplet is sucked into the syringe itself, it may be unable to push the droplet out again. One can easily practice this with PBS droplets, verifying that the droplet can be pushed back out of the needle.)
2. Using an isoflurane chamber (3% isoflurane, 1-2 L/min flow rate), induce light anesthesia in the mouse (4 postnatal weeks), 2-3 min total exposure time. Transfer the mouse to a warmed heating pad, ventral side up. If the mouse wakes up during the following steps, transfer back to the isoflurane chamber to reintroduce light anesthesia.
3. (If necessary, for hairy skin injections) Trim hair over injection site with clippers.
4. While holding the hindpaw/hindlimb in place with one hand, perform superficial subcutaneous of Dil solution with other hand:
 - a. With the bevel facing up, insert needle tip into injection site (ventral hindpaw, or ventral proximal hindlimb) until the bevel face sits completely under the skin. Apply gentle upward pressure to assure injection is very superficial.
 - b. Push Dil solution out of the needle.
 - c. Hold for 15-30 seconds to allow diffusal of solution.
 - d. Slowly remove the needle, clean any leaked solution with a Kimwipe.
5. Allow the animal to awaken on the heating pad.
6. Wait 7 days for retrograde transport of Dil.

Tissue preparation and histology:

1. Euthanize the mouse with CO₂, then perfuse with ~5-10 mL chilled PBS, followed by 10-20 mL chilled, freshly prepared 4% paraformaldehyde (PFA) in PBS.
2. Prepare and harvest skin:
 - a. (For hairy skin injection sites) Trim hair from the relevant area using clippers and/or scissors, then clean with PBS. Fully de-hair the area with Nair, then clean again with PBS.
 - b. Dissect large area of skin surrounding injection site.
 - c. Remove subcutaneous fat and connective tissue.
 - d. Pin the skin in a Sylgaard-coated dish, inside-up.
3. Post-fix the skin in chilled, fresh 4% PFA/PBS for 2h at 4°C.
4. Remove the spinal column and dissect out L3-L5 DRGs from the relevant side of the mouse's body.
5. Transfer the dissected DRGs to eppendorf tubes and post-fix the DRGs in chilled, fresh 4% PFA/PBS for 2 h at 4°C.
6. After post-fixation, transfer skin and DRGs to PBS.
7. Wash skin and DRGs 3X10 min in PBS.
8. Cryoprotect DRGs in 30% sucrose/PBS overnight at 4°C.
9. Freeze DRGs in OCT using a dry-ice ethanol bath:
 - a. Carefully mark the location of each small DRG on the embedding mold, possibly placing each DRG in a separate corner of the embedding mold, and push the DRG to the bottom of OCT before freezing. This will help in locating the DRG in the tissue block during serial sectioning.
10. Serially cryosection DRGs with a section thickness of 20-30 microns.

Imaging and analysis - skin:

1. Mount skin in PBS on a slide, using drops of vacuum grease to hold a cover slip above the skin.
2. Image red channel fluorescence on a fluorescent microscope.
3. Use ImageJ to quantify red fluorescent skin area:
 - a. Choose a background area well outside of red fluorescent injection area, measure pixel intensity mean and standard deviation (Analysis > Set Measurements > Mean gray value and Standard Deviation)
 - b. Threshold image at the mean + 10*SD background intensity level.
 - c. Measure the area of the image above the threshold level surrounding the injection site, using the freehand selection tool.

Imaging and analysis - DRGs:

1. Once DRG cryosections have fully dried (overnight at room temperature), wash sections 3X10 min in PBS to remove OCT.
 - a. It is important that Dil containing sections are not treated with detergent (TritonX100, Tween, etc) which will remove Dil fluorescence. Native fluorescence of fluorescent proteins (GFP, etc) can be imaged alongside Dil, for example if mice carry the *Mrgprd*^{EGFPf} allele or similar.
2. Mount tissue slides with Fluormount-G mounting medium.
3. Image red channel fluorescence of all sections.
4. Using ImageJ, count Dil positive cells, comparing adjacent sections to avoid double-counting labeled cells.

How to cite: (Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Olson, W. and Luo, W. (2021). Retrograde Dil labeling. Bio-protocol Preprint. bio-protocol.org/prep741.
2. Olson, W., Abdus-Saboor, I., Cui, L., Burdge, J., Raabe, T., Ma, M. and Luo, W. (2017). Sparse genetic tracing reveals regionally specific functional organization of mammalian nociceptors. eLIFE. DOI: [10.7554/eLife.29507](https://doi.org/10.7554/eLife.29507)

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